

In the Specification:

Please amend the specification as shown:

Please delete the paragraphs on page 4, lines 12-18 and replace them with the following paragraphs:

Figure 4 shows the division of nucleic acid constructs into two groups with non-

complementary ends. Figure 4 discloses the 3' to 5' "Group A ULE Linkers" sequences as SEQ ID NOS 24-29, respectively, in order of appearance and the 5' to 3' sequences all as SEQ ID NO: 23, the 3' to 5' "Group B ULE Linkers" sequences as SEQ ID NOS 30-35, respectively, in order of appearance and the 5' to 3' sequences all as SEQ ID NO: 23 and the remaining 3' to 5' sequences as SEQ ID NOS 36-39, respectively, in order of appearance and the 5' to 3' sequences all as SEQ ID NO: 23.

Figure 5 shows division using three base permutational tails. Figure 5 discloses the 3' to 5' "Group C ULE Linkers" sequences as SEQ ID NOS 40-47, respectively, in order of appearance and the 5' to 3' sequences all as SEQ ID NO: 23 and the 3' to 5' "Group D ULE Linkers" sequences as SEQ ID NOS 48-55, respectively, in order of appearance and the 5' to 3' sequences all as SEQ ID NO: 23

Figure 6 shows that the oxidation of RNA prevents primer-independent cDNA synthesis by Reverse Transcriptase. Figure 6 discloses "dT₂₄" as SEQ ID NO: 56.

Please delete the paragraphs on page 4, line 24 to page 5, line 6 and replace them with the following paragraphs:

Figure 8 shows the relationship between the efficiency of dG-tailing and the number of rNTPs at the 3' end. Figure 8 discloses "PRO-T₂₄," "PRO-T₂₃U₁" and "PRO-T₂₁U₃" as SEQ ID NOS 9-11, respectively.

Figure 9 shows the effect of primers with ribonucleotides on RNA yields. Figure 9 discloses "PRO-T₂₄" and "PRO-T₂₁U₃" as SEQ ID NOS 9 and 11, respectively.

Figure 10 shows the effect of 2' analogues on dG-tailing. Figure 10 discloses

"PRO-T₂₄," "PRO-T₂₂Fl₂," "PRO-T₂₁Fl₃" and "PRO-T₂₁OMe₃" as SEQ ID NOS 9, 12, 11 and 11, respectively.

Figure 11 shows the effect of primers with 2' analogues on RNA yields. **Figure 11 discloses "PRO-T₂₄," "PRO-T₂₁Fl₃" and "PRO-T₂₁OMe₃" as SEQ ID NOS 9, 11 and 11, respectively.**

Figure 12 shows the effect of primers with 2' analogues on RNA yields using multiple targets. **Figure 12 discloses "PRO-T₂₄" and "PRO-T₂₁Fl₃" as SEQ ID NOS 9 and 11, respectively.**

Figure 13 shows the effect of primers with 2' analogues on RNA yields using multiple targets after a process that did not involve Terminal Deoxyribonucleotidyl Transferase addition. **Figure 13 discloses "PRO-T₂₄" and "PRO-T₂₁Fl₃" as SEQ ID NOS 9 and 11, respectively.**

Please delete the paragraph on page 16, line 23 to page 17, line 13 and replace it with the following paragraph:

Additionally, when polymeric sequences are being used as primer binding sites, a common modification of this system is the use of so-called "anchored primers". In this variation, the primers comprise a set of primers with permutational identities of the base or bases at the 3' ends. Thus for example, if poly A sequences are the desired targets, a set of 24-mers with the formula 5'-T₂₃N₁-3' (**SEQ ID NO: 4**) where N₁ is G, A or C can be used as primers. Thus rather than being a single primer sequence, a primer set is used that comprises a mixture of three oligomers: 5'-T₂₃ G-3' (**SEQ ID NO: 5**), 5'-T₂₃A -3' (**SEQ ID NO: 6**) and 5'-T₂₃C-3' (**SEQ ID NO: 7**). More than one position may also be used for anchoring. For example, a set of 24-mer primers could be used with the formula 5'-T₂₃N₁ N₂-3' (**SEQ ID NO: 8**) where N₁ is G, A or C and N₂ is T, G, A or C. This formula would describe a set of 12 different primer sequences (3 x 4 permutations). By using sets of anchored primers, priming events can preferentially take place at the junction where a poly A tail had been appended to the discrete nucleotide sequence of a eucaryotic mRNA. This is in contrast to a completely homopolymeric oligo T where priming events could take place at any segment of the poly A tail. The present invention may make use of this system by using nucleotides or nucleotide analogues with the above described properties as the terminal permutational nucleotides. Thus for example, in a set with the formula 5'-T₂₃N₁-3', N₁ (**SEQ ID NO: 4**) could be rG, rA or rC. In another example, a set with the formula 5'-T₂₃N₁

N₂-3' (**SEQ ID NO: 8**) could have the permutational base identities described above, but the particular nucleotides used for N₁ and N₂ are 2' Fluoro analogues.

Please delete the paragraph on page 31, lines 6-29 and replace it with the following paragraph:

On the day of use, a 200 mM stock solution of sodium periodate (Sigma-Aldrich, St. Louis, MO) was prepared in nuclease-free water (Ambion, Austin, TX). To prepare a working solution, 10µl of 200 mM sodium periodate was added to 990 µl of 10 mM sodium acetate. 100 µg of total RNA from human liver (Stratagene, La Jolla, CA) in nuclease-free water was mixed with an equal volume of 2mM sodium periodate in 10mM sodium acetate, incubated for 60' on ice and protected from light. The RNA was purified using an RNeasy column (Qiagen, Valencia, CA) according to the manufacturer's protocol. The integrity of the modified RNA was verified by agarose gel electrophoresis and staining with SYBR Gold (Molecular Probes, Eugene, OR). First strand cDNA was synthesized from 10µg of modified and un-modified human liver RNA templates using Superscript II RNaseH⁻ reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications, in the absence or presence of dT₂₄ primer (**SEQ ID NO: 56**). RNA templates were removed by alkaline hydrolysis in 0.6M NaOH, 4mM EDTA (2x) at 37°C for 15' followed by neutralization with 6M acetic acid (20x). cDNA was then purified on MinElute columns (Qiagen, Valencia, CA) and eluted in 10µl buffer EB following the manufacturer's directions. DNA electrophoresis was carried out in a 1% agarose 0.5xTBE gel, stained with SYBR Gold and viewed with a Digital Image Station (Kodak, Rochester, NY).

Please delete the paragraph on page 32, lines 7-21 and replace it with the following paragraph:

Tailing reactions (5µl) were carried out with 100 pmoles of dT₂₁ oligonucleotide primers (**SEQ ID NO: 57**) in 1x TdT Buffer (Enzo Life Sciences, Farmingdale, NY) and 1mM cobalt chloride for 60' at 37°C, in the absence or presence of 1mM UTP and 15U Terminal Deoxynucleotidyl Transferase (TdT). The source of TdT was a recombinant bovine clone developed at Enzo Life Sciences (Farmingdale, NY) using standard methods to define the number of units of TdT activity. At the end of the tailing reaction, volumes were then increased to 50µl with the addition of 1XTdT buffer and cobalt chloride in the absence or presence of 1mM dGTP and 15U TdT. After a 15' incubation at 37°C, the reactions were quenched with 1µl of 0.5M EDTA and placed on ice. Aliquots (5µl) of each reaction were mixed with 5µl of Gel Loading Buffer II (Ambion, Austin, TX), denatured by heating for 10' at 70°C and nucleic acid strands separated by 15% PAGE containing 7.5 M Urea. A dT₂₄ primer (**SEQ ID NO: 56**) (lane 7) and a 38 bp mixed

sequence primer (lane 8) are included as size markers. Nucleic acids were visualized by staining with SYBR Gold and quantified with the Digital Image Station.

Please delete the paragraphs on page 33, line 8 to page 35, line 17 and replace them with the following paragraphs:

Since the product of the TdT mediated addition of U to the primers in the example above probably represents a collection of primers with different numbers of ribonucleotides present instead of a single discrete species, primers were synthesized with either 1 or 3 ribonucleotides already in place at the 3' end. Phosphoramidites for inclusion of ribonucleotide moieties were obtained from Glen Research (Sterling, VA). The sequences of these primers are as follows, where the 5' end comprises a T7 RNA polymerase promoter sequence and the 3' end is complementary to a poly A segment:

PRO - T₂₄ (SEQ ID NO: 9) =

5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG TTT TTT TTT TTT TTT TTT TTT TTT-3'

PRO - T₂₃U₁ (SEQ ID NO: 10) =

5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG TTT TTT TTT TTT TTT TTT TTT TTU-3'

PRO - T₂₁U₃ (SEQ ID NO: 11) =

5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG TTT TTT TTT TTT TTT TTT TTT UUU-3'

Tailing reactions (8µl) were carried out with 80 pmoles of either PRO-T₂₄ (SEQ ID NO: 9), PRO-T₂₃U₁ (SEQ ID NO: 10) or PRO-T₂₁U₃ (SEQ ID NO: 11) in 1x TdT buffer, 1 mM cobalt chloride and 24U TdT (SEQ ID NO: 19) for 15' at 37°C, in the presence or absence of 0.05mM dGTP. Aliquots of each reaction were denatured as described in Example 2 and the nucleic acids were separated by 10% PAGE containing 7.5 M Urea. Nucleic acids were visualized by staining with ethidium bromide and quantified with the Digital Image Station.

Results

The results of this experiment are shown in Figure 5, where the gel results are shown in the top part. As described above, this gel was then digitally quantified and the averaged results are

shown in the lower image. As a control, lanes 1, 3, 5, 7 and 9 show the size positions of the oligonucleotides in the absence of dGTP. As expected, the normal primer (PRO-T₂₄) (SEQ ID NO: 9) shows extensive addition of dG by TdT (lane 2 compared to lane 1). On the other hand, the chimeric oligonucleotides (carried out in duplicate) are not as efficient substrates. For instance, it can be seen in lanes 4 and 6 that even with a single ribonucleotide at the 3' end (PRO-T₂₃U₁) (SEQ ID NO: 10) there is extensive blockage of terminal addition, and the majority of the oligonucleotides remain in the position of the untreated oligonucleotide. In lanes 8 and 10, this effect can be seen to be intensified by the use of multiple ribonucleotide substitutions (PRO-T₂₃U₁) (SEQ ID NO: 10), where it can be seen that there is little if any addition by TdT.

Example 4

Chimeric primers with ribonucleotides at the 3' end show increased transcription.

(a) 1st strand synthesis

A recombinant clone (ATCC 87482) for poly A⁺ bacterial Lys A was obtained from the American Tissue Culture Collection (Manassas, VA) and used to prepare mRNA *in vitro* using standard procedures. 1µg of purified polyA⁺ Lys A RNA (1,272 nt) was mixed with 50pmole of the PRO-T₂₄ primer (SEQ ID NO: 9) or the PRO-T₂₁U₃ chimeric primer (SEQ ID NO: 11) (used in Example 3) in a 10µl solution, heated for 10' at 70°C, followed by the addition of 9µl of a RT premix containing 2 µl of 10X Stratascript RT Buffer (Stratagene, LaJolla, CA), 1µl of 10mM dNTP mix, 2µl of 100mM DTT, 1µl of 30U/µl RNase Inhibitor (Eppendorf, Boulder, CO) and 3µl of nuclease-free water. After a 3' preincubation at 42°C, 1µl of 200U/µl StrataScript RNaseH⁻ reverse transcriptase (Stratagene, LaJolla, CA) was added and the reactions were incubated at 42°C for 60' to synthesize first-strand cDNA. The final concentrations of components in the 20µl reaction mixtures were; 50mM Tris (pH 8.3), 75mM KCl, 3mM MgCl₂, 10mM DTT, 0.5mM of each dNTP, 1.5 U/µl RNase Inhibitor, 2.5 * M primer and 50ng/µl RNA. After heat inactivation of the RT (70°C for 10'), 1µl of 5U/µl RNaseH (New England Biolabs, Beverly, MA) was added and samples incubated for 20' at 37°C. First strand cDNA was purified by MinElute columns using the manufacturer's protocols. The eluted first-strand cDNA (20µl) was dG-tailed for 15' at 37°C in a 40µl reaction containing 30U TdT (SEQ ID NO: 18), 0.5mM dGTP, 1X TdT buffer and 1mM cobalt chloride. The 3'-ends of the tailed first-strand cDNA were blocked by the terminal addition of dideoxy-ATP with TdT. In this case, 40µl of termination mix containing 1 xTdT buffer, 20mM MgCl₂, 5mM dideoxy-ATP (Amersham Biosciences, Piscataway, NJ) and 15 U TdT (SEQ ID

NO: 22) was added and the samples incubated for 15' at 37°C. The single-stranded, G-tailed and terminated cDNA was purified by MinElute columns and eluted with 20µl EB.

Please delete the paragraphs on page 36, line 28 to page 38, line 28 and replace them with the following paragraphs:

Phosphoramidites for inclusion of 2' Fluoro and 2' O-methyl U analogues and universal support were obtained from Glen Research (Sterling, VA). The sequences of these primers are as follows, where the 5' end comprises a T7 RNA polymerase promoter sequence and the 3' end is complementary to a poly A segment:

PRO - T₂₁OMe₃ (SEQ ID NO: 11)

5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG TTT TTT TTT TTT TTT TTT
TTT XXX-3'

where X = 2'-O-Me-U

PRO - T₂₁FI₃ (SEQ ID NO: 11)

5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG TTT TTT TTT TTT TTT TTT
TTT YYY-3'

where Y = 2'-FI-U

PRO - T₂₂FI₂ (SEQ ID NO: 12)

5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG TTT TTT TTT TTT TTT TTT
TTT TYY-3'

Where Y = 2'-FI-U

Tailing reactions (8µl) were carried out with 50 pmoles of PRO-T₂₄ (SEQ ID NO: 9) (from Example 3), PRO - T₂₂FI₂ (SEQ ID NO: 12), PRO - T₂₁FI₃ (SEQ ID NO: 11) or PRO - T₂₁OMe₃ (SEQ ID NO: 11) in 8µl reaction mixtures containing 1x TdT Buffer, 1 mM cobalt chloride, 0.05 mM dGTP, 24U TdT (SEQ ID NO: 19), for 15' at 37°C. As controls, reactions were carried out as above with the addition of EDTA to 31mM. Aliquots of each reaction were denatured as described previously and nucleic acids were separated by 10% PAGE containing 7.5 M Urea. Nucleic acids were visualized by staining with ethidium bromide.

Results

The results of this experiment are shown in Figure 7. The reactions with EDTA serve as negative controls to show the positions of the oligonucleotides in the absence of terminal addition. In the positive control with a standard primer, there can be seen a substantial shift in position after dG addition (lane 1 compared to lane 2). However, with either 2 (lane 3) or 3 (lane 5) substitutions with 2'fluoro analogues, there is a substantial drop in the efficiency of dG addition with the 3 substitutions (PRO - T₂₁Fl₃) (SEQ ID NO: 11) being slightly more effective for blockage than the 2 substitutions (PRO - T₂₂Fl₂) (SEQ ID NO: 12). In this example, another 2' analogue was also tested. Lane 7 shows that the 2' O-methyl substitution also showed inhibition of TdT tailing.

Example 6

Transcription products from extended 2'-Fluoro or 2'-O-Methyl chimeric primers.

(a) 1st strand synthesis

20 pmoles of PRO-T₂₄ (SEQ ID NO: 9) (from Example 3), PRO - T₂₁Fl₃ (SEQ ID NO: 11) (from Example 5) or PRO - T₂₁OMe₃ (SEQ ID NO: 11) (from Example 5) primers were annealed to 0.5 µg poly A⁺ LysA (described in Example 4) in a 10µl reaction volume. First-strand cDNA synthesis was then carried out as described previously in Example 4, except that the 3' preincubation step was omitted and the RT was included in a 10µl RT Mix. Removal of RNA was achieved by base hydrolysis, (0.3 mM NaOH and 2 mM EDTA for 20' at 37°C) followed by neutralization with acetic acid. After purification through MinElute columns, the first-strand cDNA was tailed and terminated as described in Example 4., except that dCTP (0.5mM) was substituted for dGTP and only 2.5 units of TdT was used.

(b) 2nd strand synthesis

After purification, the dC-tailed, terminated first strand cDNA (18 µl) was annealed with 20 pmoles of dG₁₂ primer (SEQ ID NO: 13) (2µl) and second-strand cDNA was synthesized as described in Example 4, except that the final reaction volume was 30µl instead of 20µl.

Please delete the paragraph on page 39, line 23 to page 40, line 10 and replace it with the following paragraph:

20 pmoles of either the T7 promoter-dT₂₄ (SEQ ID NO: 9), or the T7 promoter-dT₂₁2'-F-dU₃ (SEQ ID NO: 11) primer were annealed to 10 ng of four target poly-A⁺ RNA transcripts of 750_ (SEQ ID NO: 14), 1000 (SEQ ID NO: 15), 1400 (SEQ ID NO: 16) and 2000 (SEQ ID NO: 17) nts, (10 ng each) in 10µl volumes. First strand cDNA synthesis was carried out as described in

Example 6. RNA was removed as described in Figure 6, and after purification through MinElute columns the first strand cDNA was tailed with dCTP rather than dGTP. After termination with ddATP and purification, the dC-tailed, terminated first strand cDNA (18 μ l) was annealed with 20 pmoles of dG₁₂ primer (SEQ ID NO: 13) (2 μ l) and second strand cDNA synthesis was carried out as described in Example 6, except that complete Exo⁻ Pol I was used instead of Klenow. One quarter of the double-stranded cDNA was subjected to an in vitro transcription (IVT) reaction at 37°C for 4 h using the BioArray High-Yield RNA Transcript Labeling Kit (Enzo) with unlabeled nucleotides (3.75mM of each NTP). RNA was purified by the RNeasy Kit according to the manufacturer's instructions. One-tenth of each of the eluted RNAs was denatured at 65°C in a formamide loading buffer, electrophoresed on a 1.2% agarose gel (0.5 xTBE), stained with ethidium bromide, visualized with a Kodak Image Station and quantified using Kodak software. Each individual band was measured separately.

Please delete the paragraph on page 40, line 24 to page 41, line 7 and replace it with the following paragraph:

20 pmoles of either the T7 promoter-dT₂₄ (SEQ ID NO: 9), or the T7 promoter-dT₂₁2'-F-dU₃ (SEQ ID NO: 11) primer were annealed to 10 ng of four target poly-A⁺ RNA transcripts of 750 (SEQ ID NO: 14), 1000 (SEQ ID NO: 15), 1400 (SEQ ID NO: 16) and 2000 nts (SEQ ID NO: 17), (10 ng each) in 10 μ l volumes. First strand cDNA synthesis was carried out as described in Example 6. Second strand synthesis and purification of double-stranded cDNA was performed according to a protocol in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Santa Clara, CA) for a modified version of the original Eberwine method. One quarter of the double-stranded cDNA was subjected to an in vitro transcription (IVT) reaction at 37°C for 4 hours using the BioArray High-Yield RNA Transcript Labeling Kit (Enzo) with unlabeled nucleotides (3.75mM of each NTP). RNA was purified by the RNeasy Kit as suggested by the manufacturer. One-tenth of each of the eluted RNAs was denatured at 65°C in a formamide loading buffer, electrophoresed on a 1.2% agarose gel (0.5 xTBE), stained with ethidium bromide, visualized with a Kodak Image Station and quantified using Kodak software.

Please delete the paragraphs on page 41, line 20 to page 42, line 9 and replace them with the following paragraphs:

In this example two primers will be used that have the following sequences:

The primer for first strand synthesis U₂₄ (SEQ ID NO: 20) would be comprised entirely of ribonucleotides:

U₂₄ (SEQ ID NO: 20) =

5' UUU UUU UUU UUU UUU UUU UUU UUU 3'

The primer for second strand synthesis would comprise an RNA promoter sequence and comprise the sequence:

PRO – G₁₂ (SEQ ID NO: 21) =

5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG GGG GGG GGG G-3'

A series of reactions could be carried out as described in Example 4 where **U₂₄ primer (SEQ ID NO: 20)** was used for 1st strand synthesis and **PRO – G₁₂ primer (SEQ ID NO: 21)** would be used for 2nd strand synthesis. Also, instead of the G-tailing carried out in Example 4, dCTP would be substituted to form an oligo C 3' tail to allow a subsequent binding by the **PRO – G₁₂ primer (SEQ ID NO: 21)** to carry out 2nd strand synthesis. By having the primer in the 2nd strand primer instead of the 1st strand primer as used in Example 4, transcription would proceed from the opposite direction and RNA would be transcribed that would be in the same orientation as the original target mRNA instead of the anti-sense product made in Example 4.